Utilization of an Immunostaining Technique to Demonstrate Heterogeneity in the Content of Dihydrofolate Reductase in Peripheral Blast Cells from a Patient with Acute Lymphocytic Leukemia

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INTRODUCTION

MTX has been used for many years for the treatment of leukemia. Many patients developed resistance to MTX, but the exact mechanism of drug resistance in the clinic is not clear. Based on cell culture and animal studies, it has been reported that the mechanism of resistance to MTX could be due to a decreased rate of uptake of MTX, increasing levels of DHFR, alteration of the properties of DHFR, or a combination of these mechanisms. A major difficulty in evaluating the mechanism responsible for the resistance of tumor cells to MTX may be the heterogeneity of the tumor cells. It is conceivable that some of the cells may have more DHFR content than do other cells. Therefore, even a small number of cells with high DHFR content in a tumor population could be sufficient to observe clinical resistance to MTX treatment. Thus, the ability to estimate the DHFR content in each single cell among the tumor population could be important. The use of the fluorescein-conjugated MTX to estimate the intracellular level of DHFR has been reported. Unfortunately, the poor transport of the fluorescent material and the requirement of a cell sorter limit the development of such a method. In this communication, we report the development of a methodology which utilizes a monospecific antibody to demonstrate the heterogeneity of DHFR content in the blast cells of leukemic patients.

MATERIALS AND METHODS

L1210 DHFR antibody was graciously provided by Dr. Sheila Smith of Wellcome Research Laboratories, Research Triangle Park, NC. MTX was a gift of Dr. R. L. Capizzi of the University of North Carolina at Chapel Hill, Chapel Hill, NC. All reagents not specified were purchased from Sigma Chemical Company, St. Louis, MO and were reagent grade or better. Sodium dodecyl sulfate, acrylamide, and bisacrylamide were purchased from Bio-Rad Laboratories, Richmond, CA. Sheep anti-rabbit γ-globulin, RPMI 1640 medium, and fetal calf serum were purchased from Grand Island Biological Co., Grand Island, NY. Rabbit anti-peroxidase horseradish antiserum was purchased from the United States Biochemical Corporation, Cleveland, OH.

Cells. MTX-resistant cells were developed and grown as described previously. The activity of DHFR in these cells and their sensitivity to MTX were also described. Cultured human KB cells were grown in RPMI 1640 medium supplemented with 5% fetal calf serum and kanamycin, 0.1 mg/ml.

Fresh samples of peripheral blood or bone marrow from a leukemic patient were treated with hypotonic lysis (3 times) to obtain preparations of WBC. Differential WBC were >90% blasts and lymphocytes in all cases. Nine volumes of H2O were added to the noncoagulated blood cells, followed by the immediate addition of 1 volume of 9% NaCl solution. The cells were centrifuged at 3000 rpm for 10 min, and the supernatant was removed.

**Immunostaining Procedure for Cells.** Approximately 2 x 10⁶ cultured cells or lymphocytes of leukemic patients were fixed in 2 ml of 100% methanol for 10 min. The cells were then washed twice in PBS and resuspended in approximately 0.2 ml of PBS. One or 2 drops of the cell suspension were placed on a glass slide containing a smear of albumin glycerol. The slide was placed on a warming plate at 40-50°C until the cell preparation was dry. The cells were covered with 0.1 ml of 2.5 A of antibody L1210 DHFR IgG fraction, which was diluted 1:100 in PBS containing 0.1% bovine serum albumin. They were incubated for 1 hr at room temperature and incubated at room temperature. After 18 hr, the slides were rinsed twice for 30 sec in PBS and covered with 0.1 ml of a 1:100 dilution of sheep anti-rabbit γ-globulin. The slides were incubated in a moist environment for 15 min at room temperature. After the incubation, the slides were rinsed as described above, and the cell smear was covered with 0.1 ml of a 1:100 dilution of rabbit anti-peroxidase horseradish antiserum. After a 15-min incubation at room temperature, the slides were rinsed with PBS, the cell smear was covered with 0.1 ml of 1% Triton X-100, and the cell smear was washed with 0.2 g/1iter. The slides were incubated for 10 min, followed by rinsing in PBS. The slides were immersed in 200 ml of freshly prepared solution containing 50 mM Tris-HCl, pH 7.4, 75 mg of diaminobenzidene, and 13.4 µ of 30% H₂O₂. After 30 min, the slides were rinsed twice in the 50 mM Tris-HCl buffer, first for 10 dips and then for 5 min. The slides were then exposed to vapor from 2% OsO₄ solution for 10 min, followed by rinsing in PBS. They were counterstained with 0.2% aqueous alcoholic uranyl acetate for 10 min, followed by rinsing in distilled water; and dried in air. They were then examined in a Phillips EM 301 electron microscope.
terstained by immersing the slides in a 0.01% (w/v) solution of toluidine blue O solution for 1.5 min. The slides were then dehydrated by immersing the slides in 50% ethanol for 1 min, 70% ethanol for 1 min, 95% ethanol for 10 dips, absolute ethanol for 10 dips, and xylene for 1 min. Coverslips were attached using Permount. As a control, for each slide exposed to antibody, a duplicate slide was exposed to the IgG fraction of normal rabbit serum which had not been immunized with DHFR. Based on our experience, the intensity of cell staining does not vary from day to day or with different batches of reagents. We are currently investigating whether the intensity varies with the growth stage of cultured cells when harvested.

**Properties of Antibody against DHFR.** To determine whether the L1210 DHFR antibody was monospecific, gel electrophoresis (10) was performed on equal amounts of protein from crude cell extracts of KB, KB/6b (a cloned KB subline which contains a 40-fold increase in the level of DHFR), and a sample of purified DHFR from the KB/6b cell line. After staining the gel for protein (11), the band corresponding to the purified DHFR was larger and more intense in the KB/6b than in the KB cells. A Western blot onto nitrocellulose paper (14) was performed on a duplicate gel. Using the L1210 DHFR antibody, the nitrocellulose paper was immunostained. The results showed the antibody to be monospecific to DHFR and also showed a greater intensity of staining in the KB/6b band. The details are described in our previous publication (3).

**RESULTS AND DISCUSSION**

**Immunostain of DHFR in Cells Containing Various Amounts of DHFR.** In order to determine that the DHFR antibody could be used to quantify the content of DHFR in cells, cell smears were prepared using unc loned KB cells which had different amounts of DHFR as a result of exposure to increased concentrations of MTX (3). The immunoproc edure was performed on these cells as described in “Materials and Methods.” As the DHFR content increased in the cells, the intensity of the staining increased. It should be noted that the intensity of the staining of each individual cell is not the same, indicating the heterogeneity of DHFR content in these cells (Fig. 1). When the cloned cells (3) were used, the staining was more homogeneous (Fig. 2). This observation indicated a good correlation of DHFR content and the intensity of staining.

**Immunostain of DHFR in Peripheral Blast Cells.** In an attempt to examine the content of DHFR in tumor cells of a patient undergoing MTX treatment, the immunostaining procedure was performed on the blasts of a patient with relapsed acute lymphocytic leukemia. In 1979, prior to the relapse, the patient was put on a program consisting of 10 drugs. At the time, the patient received a low dose of MTX for 4 consecutive days (10 mg/sq m p.o.). In March 1982, the patient was treated with increasing amounts of MTX (100 to 250 mg/sq m i.v.) with vincristine and asparaginase over a 3-month period. Initially, low levels of staining were seen but, as the treatment progressed, subsequently higher levels of heterogeneous staining appeared (Fig. 3). Although the 2 pictures shown in Fig. 3 did not have the same cell number, when an equal number of cells were compared, the percentage of cells with high-intensity staining was greater in the sample taken later during MTX treatment. The staining intensity of several cells from this patient’s (May 1982) preparation appears to be higher than that of the MTX-resistant line, KB/6b, while other cells in the same population appear to have a DHFR content similar to that of the KB cell line. Since DHFR derived from KB/6b and lymphoblast cells from patients share similar kinetic properties (2), the antibodies used would be monospecific to DHFR in either cell line. Furthermore, staining with premunone serum gives the same background intensity. The content of DHFR in a given cell could be semiquantitative, based on the intensity compared to KB/MTX cells with a known DHFR content.

As a control, duplicate samples were exposed to premunone rabbit serum. The intensity of staining was less than that for the cells exposed to serum containing L1210 DHFR antibody (data not shown). Based on the differential staining of cells in the sample exposed to serum containing antibody, their sensitivity to MTX could be anticipated to be different. This demonstrated clearly that the content of DHFR in each tumor cell of the population was different and that the increased level of DHFR could be one of the mechanisms of MTX resistance in the clinic. The limitation of this technique is that, in spite of the monospecificity of the DHFR antibody, this technique could not distinguish between the active, altered, or inactive DHFR in cells, and the quantitation of DHFR in cells is not precise. With proper standard cell lines, which have different contents of DHFR and different sensitivities toward MTX, this technique could be adapted by any laboratory. Further clinical investigation is required to determine whether the response to MTX and the increase in the frequency of cells containing more DHFR is related to MTX treatment.

**REFERENCES**


Fig. 1. Immunostaining of uncloned KB cells containing different amounts of DHFR using preimmune rabbit serum (upper four panels) and rabbit serum containing L1210 DHFR antibody (lower four panels). Details are described in "Materials and Methods." × 240.
Heterogeneity of DHFR Content in Tumor Cells

A. Normal Rabbit Serum IgG Fraction

KB/MTX 2.5 X 10^{-7} 
clone d

KB/MTX 2.5 X 10^{-5} 
clone b

B. DHFR Rabbit Antiserum IgG Fraction

KB/MTX 2.5 X 10^{-7} 
clone d

KB/MTX 2.5 X 10^{-5} 
clone b

Fig. 2. Immunostaining of cloned KB cells containing different amounts of DHFR using preimmune rabbit serum (A) and rabbit serum containing L1210 DHFR antibody (B). Details are described in "Materials and Methods." x 240.
Fig. 3. Immunostaining of peripheral blasts of a patient undergoing MTX therapy. Details are described in "Materials and Methods." x 120.
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